PYRIDINE NUCLEOTIDE DEHYDROGENASE BASED BIOSENSOR ELECTRODES

Background of the invention

5   Numerous studies have been performed in recent years into
     enzymatic oxidation and reduction reactions employing pyridine
     cofactors such as NADH (nicotinamide adenine dinucleotide).
     In order to exploit these reactions in vitro, it is necessary
     to recycle the NADH product of the enzymatic reaction and
10   regenerate the NAD^+ reactant. One highly controllable way of
     recycling the NADH component is via electrochemical oxidation.
     This also has the advantage that the monitoring of the current
     flowing in the electrolytic cell offers a convenient way of
     monitoring the reaction progress as a larger concentration of
15   NADH in the electrolyte will result in a larger oxidation
     current.

     It has been previously shown that it is possible to directly
     oxidise NADH to NAD^+ on the surface of an electrode, for
20   example by Gorton [J. Chem. Soc., Faraday Trans. 1, (1986) 82,
     1245-1258]. However, the major problem with this reaction is
     the requirement for a large overpotential to perform the
     reaction.   This results in the formation of radical NAD·
     species which combine on the electrode surface to form $(NAD)_2$
25   dimers, as shown by Jaegfeldt [Bioelectrochem. Bioenerg. 8
     (1981) 355] which contaminate the surface of the electrode and
     decrease the reactant concentration in the electrolyte. A
     further disadvantage of using a high overpotential is that
     other non-specific reagents present in the electrolyte are
30   also oxidised at the electrode surface, resulting in further
     contamination, and in measured oxidation currents not being
     proportional to the concentration of NADH. These oxidation
     current measurements then require correction to allow for

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oxidation of contaminant species.

Work has been performed which is aimed at reducing the
overpotential required to drive the NADH oxidation and hence
5   avoid these undesirable effects. One method involves the use
of electron transfer agents which can be coated onto the
electrode to facilitate electron transfer between the NADH and
the electrode surface. Compounds such as ortho-quinones,
benzoquinones, phenoxazine, phenothiazine and phenazine dyes
10   have all been used to pretreat the surface of the electrode
allowing the oxidation of NADH at lower overpotentials. US
5,122,456 describes the platination or palladation of an
activated carbon electrode surface in order to facilitate the
transfer of electrons between the electrode and the NADH/NAD$^+$
15   redox couple. US 5,866,353 describes the use of diazacyanine
mediators to facilitate the electron transfer between the
electrode surface and NADH or NADPH to regenerate NAD$^+$ or
NADP$^+$ respectively.

20   A second method of reducing the overpotential required to
oxidise NADH at the electrode has been developed. This
involves the use of an enzyme immobilised on the surface of
the electrode which mediates the oxidation of NADH to NAD$^+$.
US 5,538,867 describes the immobilisation of cytoplasmic
25   hydrogenases from a range of bacteria on the surface of an
electrode to lower the overpotential required to drive NAD$^+$
reduction. US 5,543,326 describes the modification of redox
enzymes to enhance their binding to an electrode surface.

30   It is known in the art that NAD$^+$ and NADH are the substrates
for over 350 dehydrogenase enzymes thus a method of performing
redox electrochemistry in both a controllable and reversible
manner will open up a huge range of potential amperometric

enzyme electrodes for a range of different substrates. Direct
electron transfer has proven to be an excellent way of
studying enzyme mediated redox catalysis on the surface of
electrodes. For example, direct electron transfer and high
5   catalytic rates have been demonstrated by Pershad *et al.*
[Biochemistry 38 (1999) 8992-8999] for both hydrogen oxidation
and proton reduction using *Chromatium vinosum* hydrogenase
immobilised on an electrode surface.

10   A problem remaining in all of the work performed on the
electrochemical interconversion of oxidised and reduced forms
of pyridine nucleotides is that the reaction still requires a
significant overpotential to proceed. None of the prior
methods for interconversion of pyridine nucleotides provide a
15   truly electrochemically reversible method of doing so.

Brief Description of Figures

Figure 1 - Shows a schematic of the Iλ subcomplex as a part of
20   complex I membrane bound and catalysing NADH oxidation, and
isolated and adsorbed onto an electrode, catalysing the
reversible interconversion of NADH and NAD^+.

Figure 2 - Shows a protein film voltammogram of the Iλ
25   subcomplex adsorbed onto a graphite electrode in the presence
of NADH and NAD^+.

Figure 3 - Shows a schematic of the Iλ subcomplex catalysing
NADH reduction in the presence of a second enzyme which uses
30   NAD^+ as a cofactor for converting a substrate to a product.

Figure 4 - Shows an SDS PAGE gel showing fractionation of the

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individual subunits from which the Iλ subcomplex is composed.

Figure 5 – Shows a plot of isosbestic points ($E_{NAD+/NADH}$) vs. pH
for the Iλ subcomplex adsorbed onto a graphite electrode and
5   in the presence of NADH and NAD^+.

Summary of the invention

The aim of the present invention is to provide a fully
10 electrochemically reversible method of driving a pyridine
nucleotide redox couple in either an oxidative or reductive
fashion through immobilisation of an isolated pyridine
nucleotide dehydrogenase module of an enzyme on an electrode
surface which is then brought into contact with an electrolyte
15 containing reduced and/or oxidised forms of the pyridine
nucleotide.

We have surprisingly found that the use of an isolated
dehydrogenase module derived from a holoenzyme can, when
20 applied to an electrode, mediate the interconversion of
oxidised and reduced forms of a pyridine nucleotide with
little or no overpotential required to drive the reaction in
either direction. This is entirely unexpected in the light of
the prior art in this area.
25
Accordingly the present invention provides an electrode for
the electrochemically reversible interconversion of the
oxidised and reduced versions of a pyridine nucleotide
comprising: an electrically conducting surface; and an
30 isolated pyridine nucleotide dehydrogenase module of an
enzyme; wherein said isolated pyridine nucleotide
dehydrogenase module is applied to the electrically conducting
surface. In a preferred embodiment, the isolated pyridine

nucleotide dehydrogenase module is derived from one of the
enzymes selected from the group comprising: NADH:quinone
oxidoreductase (complex I, EC 1.6.5.3 or EC 1.6.99.3), sodium
translocating NADH:quinone oxidoreductases (Na$^+$-NQR), soluble
cytoplasmic hydrogenases (for example EC 1.12.1.2) and soluble
dehydrogenases. More preferably, the isolated pyridine
nucleotide dehydrogenase module is the Iλ subcomplex of bovine
mitochondrial NADH:ubiquinone oxidoreductase. Also provided
are electrodes in which the isolated pyridine nucleotide
dehydrogenase module of an enzyme may be chemically or
physically modified such that the pyridine nucleotide
dehydrogenase activity is retained.

According to the invention, the enzyme may be applied to the
surface of the electrode by a method selected from the group
comprising: physisorbtion; ionic interaction; chemisorbtion;
hydrophobic interaction and binding in a polymer matrix.

In another embodiment, recombinant DNA methods known in the
art may be used to express the subunits of said isolated
pyridine nucleotide dehydrogenase module of an enzyme in a
host cell which may be assembled in the host cell or recovered
from a host cell and assembled in vitro.

Also described are electrodes wherein the electrically
conducting surface of the electrode may be made from a
material selected from the group consisting of: carbon; gold;
silver; platinum; palladium; tungsten; iridium and well doped
semi-conductor electrodes such as titanium oxide, indium
oxide, tin oxide or diamond. In a more preferable embodiment
the electrically conducting surface is a carbon material which
may be chosen from the group comprising: glassy carbon; highly
ordered pyrolytic graphite (HOPG); edge oriented pyrolytic

graphite; graphite.

The invention further comprises an electrochemical cell
comprising: a working electrode of the invention; a reference
5   electrode; one or more electrolytes and optionally an
auxiliary electrode. The reference electrode may be any
commonly used reference electrode for example a standard
calomel electrode or a silver/silver chloride electrode.

10 The electrolyte of the invention comprises one or more buffers
selected from the group comprising any commonly used buffers
for example sodium .acetate, potassium phosphate, MES, HEPES,
TAPS, and may optionally comprise a body fluid sample.

15 The invention also provides an electrochemical cell of the
invention further comprising: a substrate for a second enzyme;
and a second enzyme capable of converting the substrate into a
product; wherein said second enzyme of the invention may use a
pyridine nucleotide as a cofactor. In a preferred embodiment,
20 the second enzyme may be a dehydrogenase. More preferably,
the second enzyme of the invention may be selected from the
group comprising EC numbers: 1.1.1; 1.2.1; 1.3.1; 1.4.1;
1.5.1; 1.7.1; 1.8.1; 1.10.1; 1.11.1.1; 1.11.1.2; 1.12.1;
1.14.12; 1.14.13; 1.16.1; 1.17.1; 1.20.1.
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Furthermore the invention discloses a method for effecting the
electrochemically reversible interconversion of the oxidised
and reduced forms of a pyridine nucleotide in an electrolyte
containing the oxidised form and/or the reduced form of the
30 pyridine nucleotide said method comprising applying a
potential between the working electrode and the reference
electrode of an electrolytic cell of the invention.
Preferably the potential applied between the working electrode

and the reference electrode is such that the current flowing
through the electrochemical cell is proportional to the
concentration of either the oxidised or the reduced form of
the pyridine nucleotide present in the electrolyte.

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In a preferred embodiment of the method of the invention, said
potential applied between the working electrode and the
reference electrode may be an oxidative or reductive potential
converting the reduced or oxidised form of the pyridine

10   nucleotide into the corresponding oxidised or reduced form.
In these cases the method may further comprise: monitoring the
current flowing through the electrochemical cell; and relating
said current to the corresponding concentration of reduced or
oxidised pyridine nucleotide respectively using equations well

15   known in the art.

The present invention further provides a method of altering
the relative concentrations of oxidised and reduced forms of a
pyridine nucleotide in the solvent of an electrochemical cell

20   of the invention containing the oxidised and/or reduced forms
of said pyridine nucleotide wherein said method involves
applying a potential difference between the working electrode
and the reference electrode of the electrochemical cell.

25   Similarly, the present invention comprises a method of
altering the rate of conversion of a substrate to a product in
the solvent of an electrochemical cell of the invention
comprising a second enzyme which mediates the conversion of
substrate to product and uses the pyridine nucleotide of the

30   electrochemical cell as a cofactor, said method comprising,
applying a potential difference between the working electrode
and the reference electrode of the electrochemical cell.

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The present invention also provides a method for measuring the concentration of a substrate in an electrolyte of an electrochemical cell of the invention wherein said method comprises: applying a potential difference between the working
5   electrode and the reference electrode; monitoring the current flowing through the electrochemical cell; and relating said current to the concentration of the substrate.

In preferred embodiments, the pyridine nucleotide of the
10 invention may be NADH, NAD^+, NADPH or $NADP^+$.

Detailed description of the preferred embodiments

Throughout the specification, the term "isolated pyridine
15 nucleotide dehydrogenase module" is used to refer to a subcomplex, comprising one or more subunits, of an enzyme which modulates the interconversion of the oxidised and reduced forms of a pyridine nucleotide. Many enzymes can be described as comprising modular units in which certain
20 processes are localised. Many of these type of enzymes have more than one active site at which oxidation and reduction of cofactors takes place. The redox processes at these different active sites may be electrochemically coupled. Hence the oxidation of, for example, NADH in one subunit of such an
25 enzyme will produce free electrons which are transferred *via* internal electron transfer pathways to other subunits of the enzyme. In a number of enzymes, Fe-S clusters form the basis of these electron transfer pathways. The electrons are then used at the electrochemically coupled active site to reduce
30 other enzyme cofactor species. This can be thought of conceptually in electrochemical terms as two half-cell reactions which are coupled such that the electrons from one redox half-reaction are utilised in another spatially

separated half-reaction within the enzyme. In enzymes which
catalyse the oxidation of pyridine nucleotides, the oxidative
activity is often localised into a module of the enzyme
comprising one or more protein subunits. The active site

5   within the pyridine nucleotide dehydrogenase module is
generally linked to other regions of the enzyme by electron
transfer pathways.

This invention is characterised by the uncoupling of the

10 module of the enzyme in which the pyridine nucleotide redox
half-reaction occurs from other enzymatic redox half-
reactions. The deposition of the uncoupled module of the
enzyme onto an electrode surface allows the electrode to take
the place of the other half-reactions in the electron transfer

15 processes. This allows direct electron transfer between the
pyridine nucleotide redox couple and the electrode surface,
via the electron transfer pathways, without interference from
other redox reactions.

20 The subcomplex of the invention may consist of the extrinsic
arm of a transmembrane protein such as the NADH:quinone
oxidoreductases (complex I, EC 1.6.5.3 and 1.6.99.3). The
pyridine nucleotide molecule preferably refers to NADH, NAD$^+$,
NADPH or NADP$^+$. In a preferred embodiment, the isolated

25 pyridine nucleotide dehydrogenase module consists of the Iλ
subcomplex of NADH:ubiquinone oxidoreductase from bovine heart
mitochondria. A technical advantage is that the enzyme active
site is specific for the substrates NADH and NAD$^+$. It will
not react with other molecules in solution and therefore a

30 highly specific electrode can be formed at which side
reactions are negligible.

Suitable pyridine nucleotide dehydrogenase enzymes from which

the isolated modules of the invention can be derived include:
NADH:quinone oxidoreductases (EC 1.6.5.3 and 1.6.99.3) which
can be derived from both prokaryotic and eukaryotic sources;
sodium translocating NADH:quinone oxidoreductases (Na$^+$-NQR)

5   which can be typified by the enzymes from *Vibrio alginolyticus*
and *Vibrio cholerae*; and soluble dehydrogenases or soluble
cytoplasmic hydrogenases typified by that from *Ralstonia
eutrophus* (EC 1.12.1.2).

10   Enzymes of this type can be broken into their composite
modular units by methods known in the art utilising a wide
variety of disruption agents. The disruption agents used in
the present invention may be any chaotropic salts or
detergents known in the art for example dodecyltrimethyl-

15   ammonium bromide (DTAB), lauryldimethylamine oxide (LDAO) or
sodium perchlorate.

The NADH:ubiquinone oxidoreductase from bovine heart
mitochondria used in the invention consists of an assembly of

20   46 different protein subunits. Following disruption, the NADH
dehydrogenase module is isolated in solution by centrifugation
on a linear sucrose gradient followed by concentration and gel
filtration as is standard in the art and described in example
1. The isolated Iλ NADH dehydrogenase module has a structure

25   which consists of 14 or 15 protein subunits, one flavin
mononucleotide (FMN) and a chain of an estimated eight Fe-S
clusters along which electrons can be transferred. Figure 1
shows a schematic of the relationship between the
NADH:ubiquinone oxidoreductase and the NADH dehydrogenase

30   module. Figure 4 shows an SDS PAGE gel showing the number of
protein subunits which make up the Iλ subcomplex.

Smaller subcomplexes of this enzyme are known which are also useful in the present invention. An example of this is the flavoprotein subcomplex derived from NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria

5   which consists of three protein subunits, one FMN and two Fe-S clusters. The flavoprotein subcomplex comprises the 51kDa, 24kDa and 10kDa subunits of the Iλ subcomplex. The flavoprotein subcomplex may be isolated by standard methods well known in the art [Galante et al. Archives of Biochemistry

10   and Biophysics 192 (1979) 559-568] using sodium perchlorate as the chaotropic salt.

In addition to the three subunit flavoprotein subcomplex from bovine NADH:ubiquinone oxidoreductase (complex I), an over-

15   expressed enzyme comprising the prokaryotic (E.coli) homologues to the 75kDa, 51kDa and 24kDa Iλ subunits may also describe a useful embodiment of the enzyme for adsorption to the working electrode of the present invention. This three protein subcomplex may be isolated by standard methods well

20   known in the art [Braun et al. Biochemistry 37 (1998) 1861-1897]. Equivalent subcomplexes containing the NADH binding site from other organisms may also be useful in the present invention.

25   Recombinant DNA methods may also be used to express the subunits of the module in a host cell, which may be assembled in the host cell or recovered from a host cell and assembled in vitro. Recombinant DNA methods may be particularly relevant for expression of the small flavoprotein

30   subcomplexes.

The term "overpotential" is used to refer to the potential

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required over and above the equilibrium electrode potential in
order to induce a current to flow between the working
electrode and a second electrode in an electrolytic cell. In
a voltammogram with current on the vertical axis and potential

5   on the horizontal axis, the overpotential of a system can be
visualised as the substantially horizontal section of the
system response in which there is little or no current flowing
despite a variation in potential away from the equilibrium
potential.

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The term "electrochemically reversible" is used to refer to a
reaction at the working electrode of an electrochemical cell
in which little or no applied overpotential is required to
induce a current flow in the cell. In a voltammogram with

15   current on the vertical axis and potential on the horizontal
axis, this is visually displayed as a system response
characterised by the absence of a substantially horizontal
part of the response in which there is little or no current
flowing despite a variation in potential away from the

20   equilibrium potential.

Electrochemical cells for use in accordance with the invention
may be constructed according to conventional methods known as
such in the art. For example, US 5,866,353 describes

25   construction of an electrolytic cell for evaluation of
mediator compounds bound to an electrode surface. US
5,122,456 discloses apparatus for determining the amount of
NADH in solution through contacting the solution with an
activated carbon electrode.

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Typically, the electrochemical cell may be constructed
comprising a working electrode immersed in an electrolyte
solution. A reference electrode is immersed in an electrolyte

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which may or may not be the same as that in which the working
electrode is immersed. The working electrode is in electrical
contact with the reference electrode either by being immersed
in the same electrolyte solution in the same vessel or by

5   being immersed in an electrolyte which is electrically linked
to the reference electrode electrolyte by a salt-bridge or a
semi-permeable membrane.

Preferably the electrochemical cell further comprises an

10   auxiliary electrode immersed in the electrolyte. The
auxiliary electrode is in electrical contact with the working
electrode either by being immersed in the same electrolyte
solution in the same vessel or by being immersed in an
electrolyte which is electrically linked to the reference

15   electrode electrolyte by a salt-bridge or a semi-permeable
membrane.

The salt-bridge or semi-permeable membrane is in contact with
both electrolyte solutions and is permeable to charge carrying

20   species but does not allow direct mixing of the electrolytes.

Working electrodes to which the isolated pyridine nucleotide
dehydrogenase module may be applied can be selected from
electrically conducting materials commonly used in the art for

25   the construction of electrolytic cells. For example
P.N.Bartlett et al. [Bioelectrochemistry, 56 (2002) 117-122]
describes the use of carbon microelectrodes for the evaluation
of electron transfer mediators for NADH oxidation. In
addition US 6,340,597 describes the manufacture of biosensor

30   electrodes by formation of composite electrodes from electro-
conducting materials, chemical mediator compounds and a solid
binding compound.

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A working electrode may be constructed from an electrically
conducting material including carbon, gold, silver, platinum,
palladium, tungsten, iridium and well doped semiconductor
electrodes such as titanium dioxide, indium oxide, tin oxide,
5   or diamond.

In a preferred embodiment, the working electrode comprises a
carbon component the surface of which is exposed to the
electrolyte solution. The carbon component may be made from a
10   material selected from the group comprising: graphite, highly-
ordered pyrolytic graphite (HOPG), edge oriented pyrolytic
graphite, and glassy carbon. The carbon component may be a
disc shape of diameter between 0 and 100mm, preferably between
0.1 and 20mm, more preferably between 1 and 5mm, and typically
15   about 3mm. This disc may be embedded centrally into the end
of a cylinder of electrically insulating material such that
one face of the carbon disc is exposed.

The isolated pyridine nucleotide dehydrogenase module is
20   applied to the surface of the working electrode which it uses
as an electron acceptor or donor for oxidation of the pyridine
nucleotide (at high potentials) or reduction of the pyridine
nucleotide (at low potentials). In a preferred embodiment,
the isolated pyridine nucleotide dehydrogenase module is in
25   aqueous solution at between 1µM and 10mM, preferably between
5µM and 100µM, more preferably between 20µM and 50µM and even
more preferably about 30µM. This solution may be physically
applied to the electrode surface at a coverage of preferably
between 0 and 2 µl/mm^2, more preferably between 0 and 1
30   µl/mm^2, more preferably between 0.1 and 0.5 µl/mm^2, even more
preferably about 0.1 µl/mm^2 after which the solvent is allowed
to evaporate.

The isolated pyridine nucleotide dehydrogenase module may be
applied to the surface of the working electrode by simple
physical application such as immersing the electrode in a
solution of the isolated pyridine nucleotide dehydrogenase

5   module or applying a solution of said module to the electrode
surface and allowing the solvent to evaporate. This results
in the physisorbtion of the isolated pyridine nucleotide
dehydrogenase module.

10   In another embodiment, the isolated pyridine nucleotide
dehydrogenase module may be modified in such a way that the
dehydrogenase activity is retained but the chemical or
physical interaction with the surface of the working electrode
is increased. Methods of modifying enzymes are well known in

15   the art, for example US 5,543,326 describes a range of enzyme
modifications leading to an increased adhesion of the enzyme
to an electrode surface.

In use, the working electrode may be rotated to ensure that a
20   continuous supply of reactants to the electrode/electrolyte
interface is maintained. The speed of rotation of the working
electrode may be between 0 and 5000 revolutions per minute
(rpm), preferably between 100 and 3000rpm, or between 800 and
1200rpm, even more preferably about 1000rpm.
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In a preferred embodiment a laminar flow of electrolyte is
maintained across the end of the electrode cylinder.

In use, the working electrode may be operated substantially in
30   the absence of oxygen either as an atmosphere or dissolved in
the electrolyte. To achieve this, the electrode may be
operated under an atmosphere of inert gas and using a degassed
electrolyte or one purged with inert gas. The inert gas may

be any one of helium, argon, nitrogen, neon, krypton, xenon.
Preferably the concentration of oxygen in the operating
atmosphere is less than 50ppm, more preferably less than
20ppm, even more preferably less than 5ppm. This can be
5   achieved by using the electrolytic cell in an anaerobic
glovebox, which is standard apparatus in the art.

The reference electrode is a standard reference electrode a
number of which are well known in the art such as a standard
10   calomel electrode or a silver/silver chloride electrode.
Preferably the reference electrode is a standard calomel
electrode.

The auxiliary electrode comprises an electrically conducting
15   component the surface of which is exposed to the electrolyte
solution. The auxiliary electrode may be made from an
electrically conducting material selected from the group
consisting of: platinum; palladium; gold; silver; iridium;
tungsten; nickel and carbon. Preferably the auxiliary
20   electrode is a platinum wire.

In the preferred three electrode electrolytic cell, the
electrodes are electrically linked to a potentiostat [e.g. an
Autolab Electrochemical Analyzer (EcoChemie, The
25   Netherlands)], which is apparatus well know in the art, in
such a way that a potential can be applied and measured
between the working electrode and the reference electrode and
a current can flow and be measured between the working
electrode and the auxiliary electrode.
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In a two electrode system, the electrodes are similarly
electrically linked to a potentiostat, in such a way that a
potential can be applied and measured between the working

electrode and the reference electrode and a current can flow
and be measured also between the working electrode and the
reference electrode.

5   The electrolyte solution comprises a liquid containing free
charge carriers and optionally one or more pH buffers. In the
presence of pH buffers, the solution may be buffered to any pH
at which the enzymes of the invention are stable. Preferably
the solution is maintained at a pH of between 5 and 10 and
10 more preferably about pH 8. The charge carriers may be
selected from the group consisting of: protons; hydroxide
ions; halide ions; metal ions; ammonium ions and oxyanions
such as nitrate ions; phosphate ions or sulphate ions. The
liquid may be water, dimethylsulphoxide (DMSO) or acetonitrile
15 and is preferably water. Suitable pH buffers include any
buffer well known in the art and may include sodium acetate,
MES, HEPES, TAPS or potassium phosphate. The electrolyte
solution may contain any supporting electrolyte salt well
known in the art to enhance electrical conductivity of the
20 solution. In a preferred embodiment, the electrolyte solution
contains between 10 and 200mM NaCl as a supporting electrolyte
in water. More preferably, the electrolyte solution contains
about 100mM NaCl in water.

25 In an electrolytic cell, with the isolated pyridine nucleotide
dehydrogenase module adsorbed onto the working electrode, the
current flowing with the working electrode at a given
potential relative to the reference electrode, may be used as
a quantitative measure of the amount of oxidised or reduced
30 pyridine nucleotide present in the electrolyte. The current
flowing in the electrochemical cell can be related to the
concentration of the oxidised or reduced form of the pyridine
nucleotide present in the electrolyte through equations which

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are well know in the art.

Furthermore, a potential applied to the working electrode can
be used to drive the pyridine nucleotide redox couple in
5   either an oxidative fashion, through the application of a
positive potential relative to the potential of the pyridine
nucleotide couple, or a reductive fashion, through the
application of a negative potential relative to the pyridine
nucleotide couple.

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Figure 2 shows how the current flowing in the electrolytic
cell responds to the applied potential as the potential is
scanned between two limits. The current flowing can be
thought of as the catalytic rate of oxidation or reduction and
15   the potential applied can be thought of as the thermodynamic
driving force. As indicated in figure 2 scanning in the
positive potential direction results in a positive current
flowing indicating the oxidation of pyridine nucleotide.
Conversely, scanning in the negative potential direction
20   results in a negative current flowing indicating pyridine
nucleotide reduction.

In figure 2 the waveform described by the current response
(with contributions from both oxidation and reduction of the
25   pyridine nucleotide) is a single, continuous sigmoidal wave.
The absence of a horizontal part of the voltammogram with
current equal to zero, with current on the vertical axis and
potential on the horizontal axis shows that the
electrocatalytic interconversion of oxidised and reduced forms
30   of the pyridine nucleotide is electrochemically reversible.

In one embodiment the electrolyte in the electrochemical cell
further comprises a second enzyme. This second enzyme uses a

pyridine nucleotide as a cofactor in the conversion of a
substrate into a product as shown schematically in figure 3.
Reduced pyridine nucleotide is then converted back to its
oxidised form at the working electrode by the immobilised
5   pyridine nucleotide dehydrogenase module. Preferably the
conversion of substrate into product at the secondary enzyme
is the rate limiting step with respect to the oxidation of the
pyridine nucleotide.

10   In an analogous system, the second enzyme may convert the
pyridine nucleotide from its reduced form to its oxidised form
in its conversion of a substrate into a product. In this case
oxidised pyridine nucleotide is then converted back to its
reduced form at the working electrode by the immobilised
15   pyridine nucleotide dehydrogenase module.

The second enzyme may in principle be any dehydrogenase known
in the art which uses a pyridine nucleotide as a cofactor.
The second enzyme may be selected from the group comprising
20   enzymes with EC numbers: 1.1.1; 1.2.1; 1.3.1; 1.4.1; 1.5.1;
1.7.1; 1.8.1; 1.10.1; 1.11.1.1; 1.11.1.2; 1.12.1; 1.14.12;
1.14.13; 1.16.1; 1.17.1; 1.20.1.

Possible substrates for these second enzymes are well defined
25   and described in detail in the art. The range of possible
substrates for a given enzyme may be determined by reference
to EC number databases commonly used by those skilled in the
art.

30   In the embodiment comprising a second enzyme, the electrical
current measured between the working electrode and the
auxiliary electrode may be directly related to the rate of
conversion of the substrate to product at the second enzyme.

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As such, this embodiment provides a method of detecting and quantifying a variety of substrates in solution through careful selection of the second enzyme.

5   It will be apparent that electrodes of the present invention may be used in biosensor applications. Such an embodiment may provide a method for the amperometric determination of the concentration of an analyte in a biological fluid sample. This biological fluid sample may be a body fluid such as
10   blood, plasma or urine. The electrodes of the present invention are particularly suited to the analysis of biological fluids due to the absence of a significant required overpotential. This results in the minimised oxidation of other fluid components, such as vitamin C, uric acid, or
15   acetaminophen. This reduces the error in amperometric measurements from fluid samples due to the oxidation of other fluid components. Errors of this type occur especially in systems requiring a high overpotential.

20   Biosensor applications of the present electrode invention may include the direct detection of pyridine nucleotides and measurement of their concentration in solution. Further embodiments may also include the use of the second enzyme system of the invention in the amperometric measurement of
25   concentrations of other biological fluid analytes by utilising a variety of possible second enzymes. Biological fluid analytes of these embodiments may include, blood ketones, hormones such as testosterone, steroids, amino-acids notably phenylalanine, lactate, sugars, alcohols, and pharmaceuticals
30   and their metabolites. In the detection of these various analytes, a second enzyme may be chosen which converts the analyte into a product with concomitant transformation of a pyridine nucleotide from its oxidised form to its reduced form

(or alternatively from its reduced form to its oxidised form).
The original form of the pyridine nucleotide is then
regenerated at the isolated pyridine nucleotide dehydrogenase
module adsorbed onto the electrode surface. The current

5   flowing in the electrolytic cell can then be related to the
concentration of one form of the pyridine nucleotide in
solution and hence to the concentration of the second enzyme
substrate in the electrolyte.

10 A further application of the embodiment comprising a second
enzyme is to drive a reaction in solution. In this use of the
electrode, a potential is applied to the electrode to which
the isolated pyridine nucleotide dehydrogenase module is
adsorbed, such that the pyridine nucleotide in solution is

15 transformed from its reduced to its oxidised form by the
adsorbed module. The second enzyme in the system may be
selected such that it uses the oxidised form of the pyridine
nucleotide as a cofactor in transforming a substrate to a
product. The increased concentration of the oxidised pyridine

20 nucleotide, and the lack of the reduced pyridine nucleotide,
in solution then drives the second enzyme to convert
substrates to products.

In an analogous manner, the reverse reaction also represents a

25 useful embodiment of this invention. In this case, a pyridine
nucleotide in solution is transformed from its oxidised to its
reduced form by the adsorbed module on application of a
potential to the electrode to which the isolated pyridine
nucleotide dehydrogenase module is adsorbed. The second

30 enzyme in the system is then selected such that it uses the
reduced form of the pyridine nucleotide as a cofactor in
transforming a substrate to a product. The increased
concentration of the reduced pyridine nucleotide, and the lack

of the oxidised pyridine nucleotide, in solution then drives
the second enzyme to convert substrates to products.

Through the careful selection of the second enzyme, a wide
5   range of enzyme mediated reactions can be controllably driven
in solution to form useful or desirable products. In this
way, the control of the potential applied to the electrode
results in the control of the rate of reaction at the second
enzyme, i.e. the rate of formation of products.
10

Using this "driveable" second enzyme embodiment of the
invention, provides access not only to a range of desirable
products some of which may only be accessible via enzymatic
reactions, but also to a controllable method of making these
15 products.

Examples of applications using this "driveable" second enzyme
embodiment of the invention include the synthesis of optically
pure compounds which may have uses in pharmaceutical or food
20 additive industries. Embodiments of this type may include the
stereoselective synthesis of amino acids from α-keto
carboxylic acids in the presence of an ammonium salt and an
amino acid dehydrogenase. Reduction of α-keto acids or
oxidation of alcohols to give hydroxy acids mediated by either
25 the forward or reverse action of dehydrogenase enzymes may
also represent a useful embodiment of this invention.

In addition, reactions of this type may be used to produce
alcohols, aldehydes or ketones which are commonly utilised in
30 the chemical industry and in the manufacture of perfumes,
flavourings and food additives. Examples of reactions of this
type include: the reduction of ketones or aldehydes to
alcohols, and the corresponding reverse reactions, using

alcohols, and the corresponding reverse reactions, using
alcohol dehydrogenases; the conversion of sugars into alcohols
using dehydrogenase enzymes; and the transformation of diols
into chiral lactones using alcohol dehydrogenases.

The functionalisation of steroids can also be performed
enzymatically in the presence of a pyridine nucleotide and as
such represents a possible further embodiment of the present
invention. Examples of this type of reaction are typically
dehydrogenations utilising hydroxysteroid dehydrogenases.

A huge number of potential applications are envisaged using
this second enzyme embodiment of the invention. In all of the
examples of this type of embodiment, the adsorbed enzyme
module performs a similar task, that of regenerating the
oxidised or reduced form of the pyridine nucleotide which is
used as a cofactor by the second enzyme in transforming
substrates into products. It is therefore only necessary for
the adsorbed enzyme to be present in catalytic amounts to
effect regeneration of the oxidised or reduced form of the
pyridine nucleotide. It is this regeneration of the oxidised
or reduced form of the pyridine nucleotide that enables the
second enzyme to maintain turnover of substrate to products.

Example 1 - Isolation of the Iλ module from bovine
NADH:ubiquinone oxidoreductase.

An aliquot of solution containing ca. 10 mg mL^{-1} bovine
complex I, LDAO, DTAB, potassium phosphate buffer (pH 7.5) and
dithiothreitol, was layered onto a linear sucrose gradient.
This sucrose gradient was then centrifuged for 18 hours at
200,000 *g*. The sharp yellow-brown band in the center of the
gradient was collected and concentrated, and further purified

using a Superose 6 HR 10/30 gel filtration column. Fractions
spanning the apex of the symmetrical absorbance peak were
pooled and stored in liquid nitrogen.

5   SDS PAGE of this product demonstrated that this is indeed the
subcomplex Iλ. The SDS PAGE gel is shown in figure 4 with
subunits marked on the left of the gel.

Example 2 - Adsorption of the isolated Iλ module onto a
10 electrode surface and subsequent voltammetry.

Protein film voltammetry (PFV) studies were performed on the
isolated Iλ subcomplex of the invention.

15 1μL of a 30μM aqueous solution of subcomplex Iλ was applied to
the surface of a freshly polished 3mm diameter pyrolytic
graphite edge rotating-disc electrode. When the solvent had
evaporated the electrode was placed into a thermostatted all-
glass electrochemical cell. An aqueous solvent was added to
20 the electrochemical cell. The solvent contained 0.1M NaCl as
a supporting electrolyte and a mixed buffer system to control
the pH of the electrolyte solution. The mixed buffer system
consisted of 10mM sodium acetate, MES, HEPES and TAPS salts.
The pH was maintained at 7.82. NAD^+ and NADH (Roche) were re-
25 purified by standard procedures and then added to the
electrolyte each to a concentration of 1mM. A standard
calomel reference electrode was used and an auxiliary
electrode consisting of a platinum wire was inserted into the
electrolyte. The electrodes were connected to an Auto lab
30 Electrochemical Analyzer (EcoChemie). The working electrode
was rotated using an EG&G electrode rotator. The entire
apparatus was contained in an anaerobic glovebox with an .

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oxygen concentration of less than 2ppm.

The potential of the electrochemical cell was held at open
circuit for 10s before scanning. PFV was then performed at a
5   scan rate of 10mVs^{-1} at a temperature of 20°C and with an
electrode rotation rate of 1000rpm.

The voltammogram resulting from repeated scanning of the
potential between 0 and -0.6V is shown in figure 2. All
10   potentials have been corrected to the standard hydrogen
electrode scale [Bard, A.J.; Faulkner, L.R. Electrochemical
Methods: Fundamentals and Applications; Wiley: New York,
2001].

15   The single, continuous, sigmoidal waveshape of Figure 2
confirms that both NADH oxidation and NAD$^+$ reduction
are electrochemically reversible, and do not require any
overpotential.

20   No signals were observed in the absence of subcomplex Iλ.

Example 3 – Electrochemical reversibility of NADH/NAD$^+$
interconversion over a range of pH values.

25   The isosbestic points of Figure 2 (shown as $E_{NAD+/NADH}$) denote
the potential at which the rate of catalysis in the oxidative
direction is equal to that in the reductive direction. In
Figure 2 the concentrations of NADH and NAD$^+$ are equal and
therefore the isosbestic potential is the reduction potential
30   of NAD$^+$ ($E_{NAD+/NADH}$). A plot of $E_{NAD+/NADH}$ (measured using PFV,
from isosbestic potential points) as a function of pH is shown
in Figure 5. $E_{NAD+/NADH}$ varies linearly with pH, over a wide pH

range, and conforms to the predictions of the Nernst Equation
(solid line in Figure 5). This indicates that the redox system
behaves as predicted theoretically and hence is
electrochemically reversible over this wide pH range.